doi:10.1111/j.1365-2591.2008.01510.x

In vitro genotoxicity of root canal sealers

V. Brzovic[1], I. Miletic[1], D. Zeljezic[2], M. Mladinic[2], V. Kasuba[2], S. Ramic[3] & I. Anic[1]

[1]Department of Endodontics and Restorative Dentistry, School of Dental Medicine, University of Zagreb, Zagreb, Croatia;[2]Division for Mutagenesis, Institute for Medical Research and Occupational Health, Zagreb, Croatia; and [3]University Hospital for Tumors, Zagreb, Croatia

Abstract

Brzovic V, Miletic I, Zeljezic D, Mladinic M, Kasuba V, Ramic S, Anic I. *In vitro* genotoxicity of root canal sealers. *International Endodontic Journal*, **42**, 253–263, 2009.

Aim To evaluate the effect of leakage on differences in genotoxicity of root canal sealers *ex vivo* according to their main components using two different cytogenetic assays.

Methodology Six materials of different composition (GuttaFlow, Epiphany, Diaket, IRM, SuperEBA and Hermetic) were tested on human peripheral blood lymphocytes using the comet assay and chromosomal aberration analysis. Prepared materials were eluted in physiological solution for 1 h, 1 day, 5 and 30 days. Thereafter cultures were treated with 8 μg, 4 μg and 2 μg of each sealer. Frequencies of chromatide and chromosome breaks and accentric fragments were determined. Comet assay was used to evaluate primary DNA damage by measuring tail length and tail intensity. Chi-square, Fisher's PLSD (Protected Least Significant Difference) and Kruskall–Wallis non parametric tests were used for statistical analysis.

Results After 1-h elution only the highest dose of Diaket, Hermetic and SuperEBA significantly (P = 0.035, P = 0.048, P = 0.037 respectively) affected the measured cytogenetic parameters. The migration ability of DNA was more strongly affected than induction of chromosomal aberrations. After elutions longer than 24 h none of the tested sealers exhibited a genotoxic effect.

Conclusion Under the conditions used in the study all sealers had acceptable biocompatibility in terms of genotoxicity.

Keywords: chromosomal aberrations, comet assay, cytotoxicity, genotoxicity, *in vitro*, root canal sealers.

Received 27 February 2008; accepted 4 November 2008

Introduction

Root filling materials usually remain in close contact with living periapical tissues over a long period of time. The tissue's response to these materials is important and may influence the outcome of endodontic treatment. Therefore it is critical to assess the biocompatibility of dental materials using a variety of *ex vivo* and *in vivo* tests. Previous studies have shown that some groups of dental materials (zinc oxide-eugenol (ZnOE)-, resin-, polymer- and silicone-based) may induce local and systemic adverse effects due to the release of extractable monomers and/or other inorganic and organic ingredients (Spahl & Budzikiewicz 1994, Geurtsen 1998). For example, Serene *et al.* (1988) reported that ZnOE sealers activated the complement system and produced an inflammatory reaction. Non specific histocompatibility tests showed that eugenol-based sealers elicited a pronounced tissue irritation (Gulati *et al.* 1991). Furthermore, when implanted into the mandibular bone of rabbits, ZnOE sealer affected the normal concentrations of Zn in various organs due to their release from the materials (Kolokuris *et al.* 1998). Nencka *et al.* (1995) implanted a polyvinyl-based sealer into the tibia of rats and observed a severe inflammatory reaction after 3 days, with a gradual decrease in intensity until no reaction was seen after 180 days. Spangberg (1969) found that Diaket was highly toxic *ex vivo*, causing extensive tissue necrosis and long-lasting irritation. Also, Olsson *et al.* (1981) reported mild tissue

Correspondence: Marin Mladinic, Institute for Medical Research and Occupational Health, Division for Mutagenesis, Ksaverska 2, Zagreb, Croatia (Tel.: +385 1 4673 188; fax: +385 1 4673 303; e-mail: mmladinic@imi.hr).

reactions with Diaket after longer periods of time. However, polyvinyl-based materials are considered to exhibit acceptable biocompatibility. Resin-based materials were also found to cause pulp inflammations (Stanley 1992).

Many studies have reported the cytotoxicity of various root canal sealers on different cell lines *ex vivo*. Takahara *et al.* (1990) reported significant cell cycle delay on rat bone cells using Diaket and AH-26. Briseno & Willershausen (1991) using the same materials reported severe cytotoxicity in human gingival fibroblasts, whilst Torabinejad *et al.* (1995) reported a limited cytotoxicity of SuperEBA and IRM. Moreover, Asrari & Lobner (2003) detected significant neurotoxicity of SuperEBA and Diaket in murine cerebral cortical cells. All the mentioned studies focused on cytotoxicity evaluations, whilst there are no published data on their genotoxicity as one of the important factors influencing biocompatibility. Genotoxic damage will not necessarily lead to cell death or any other instantly observable event. Rather, it is damage of the cell genome that may significantly diminish the tissue's self-repairing potential or in the long term cause the development of neoplasia.

The aim of this study was to evaluate the genotoxicity of different groups of root canal sealers: ZnO-silicone-based (GuttaFlow), resin-based (Epiphany), polymer-based (Diaket) and ZnO-eugenol-based (Hermetic, IRM and SuperEBA). At the end of the elution period (1 h, 1 day, 5 days and 30 days) set materials were introduced into the lymphocyte cultures. To detect a wider range of types of genomic damage two different methods were used, chromosomal aberration analysis and comet assay.

Methods and materials

Blood sampling

Evaluation of root filling materials was performed on lymphocytes obtained from three young, healthy, non smoking voluntary donors. According to a questionnaire, which the donors completed, they had not been exposed to any physical or chemical agent that might have interfered with the results of genotoxicity testing in the 12 month period prior to blood sampling. Blood was drawn by antecubital venipuncture into heparinized vacutainers (Becton Dickenson, Plymouth, UK). All donors were acquainted with the study and they signed permissions for their blood samples to be used for scientific purposes.

Preparation of root filling materials

The study comprised genotoxicity testing of the root canal sealers described in Table 1. Under aseptic conditions 32 μg of each specific material was placed in each of 24 wells of a Culture Plate (TPP, Trasadingen, Switzerland). Each material was allowed to set as specified by the manufacturer; using the Elipar TriLight (3M ESPE, St. Paul, MN, USA) halogen curing unit (Epiphany) or by leaving them in air after the components were mixed (GuttaFlow, Diaket, Hermetic, IRM and SuperEBA). Set materials were covered with 2 mL of saline solution (NaCl 0.9%, Sigma, St. Louis, MO, USA). To test the effect of leakage of residual components on the genotoxicity of GuttaFlow, Epiphany, Diaket, Hermetic, IRM, SuperEBA by duration of elution, eluates were set up 1 h, 1 day, 5 and 30 days prior to blood sampling, so that they all ended simultaneously. At the end of each elution period the saline solution was discarded and whole blood cultures (9 mL) were treated with 8 μg, 4 μg or 2 μg of each set material.

Trypan blue exclusion test

Cultures for cytotoxicity testing were set up at the end of elution periods. Two millilitres of whole blood was

Table 1 Composition of root canal sealers comprised by the study

Root canal sealer	Composition
GuttaFlow	Polydimethylpolymethylhydrogensiloxane silicone oil, paraffin oil zirconium dioxide, platin catalyst, gutta-percha, zinc oxide, barium sulfate, nano silver
Epiphany	Mixture of UDMA, PEGDMA, EBPADMA, BISGMA resins, silane-treated bariumborosilicate glasses, barium sulfate, silica, calcium hydroxide, bismuth oxychloride, amines, peroxide, photo initator, stabilizers, pigment
Diaket	Zinc oxide, bismuth phosphate, diketone, vinyl acetate copolymer, vinyl ether copolymer, dichlorophen [2,2′-methylenebis(4-chlorophenol)]
Hermetic	Eugenol (4-allyl-2-methoxy phenol), perubalsam (benzyl esters of benzoic and cinnamic acidzinc oxide, zinc stearate, zinc acetate dehydrate, circonium oxide
IRM	Eugenol, acetic acid, zinc oxide, polymethyl methacrylate
SuperEBA	Eugenol, zinc oxide, alumina, natural resin, ortho ethoxy benzoic acid

introduced into 6 mL of F-10 HAM's medium (Sigma, St. Louis, MO, USA) without serum or mitotic activator. Cultures were treated with 32 μg, 16 μg, 8 μg, 4 μg or 2 μg of each root filling material for 48 h at 37 °C. Thereafter, the cultures were centrifuged at 1000 rpm for 10 min. Supernatant was removed and the precipitate was resuspended and placed onto 3 mL of Ficol (Sigma, St. Louis, MO, USA) so as not to mix the two phases. Samples were centrifuged at 600 rpm for 30 min. Fifty microlitres of the lymphocyte layer was mixed with 50 μL of 0.4% trypan blue (Sigma, St. Louis, MO, USA), dropped on microscope slides and covered with cover slips. Specimens were analysed using an Olympus CX 40 light microscope (Tokyo, Japan) under 100× magnification. For each concentration tested 1000 lymphocytes were analysed, by counting unstained (viable) cells. Blue coloured cells were considered to be nonviable (Newell 1998). As 32 μg and 16 μg exhibited 30% more cytotoxicity, they were excluded from further genotoxicity testing.

Chromosomal aberration analysis

In order to initiate the cell cultures, 0.8 mL of whole blood was introduced into a cell culture flask (Nunc GmbH, Wiesbaden, Germany) containing 8.2 mL of F10 medium (Sigma, St. Louis, MO, USA) supplemented with 20% foetal bovine serum (Sigma, St. Louis, MO, USA), 10 μg mL^{-1} of phytohemagglutinin (Murex, Dartford, UK), 2.5 g L^{-1} of phenol red pH indicator (Sigma, St. Louis, MO, USA), 100 IU of penicillin (Sigma, St. Louis, MO, USA) and 100 IU of streptomycin (Sigma, St. Louis, MO, USA). A 8 μg, 4 μg, or 2 μg weight of each root filling material was introduced immediately, into the cultures The same treatment scheme was used for each of four elution times (1 h, 1 day, 5 and 30 days). Simultaneously, negative control cultures were treated with the same volume of saline solution (NaCl 0.9%, Sigma, St. Louis, MO, USA); the positive controls were treated with bleomycin (Sigma, St. Louis, MO, USA) at a final concentration of 30 μg mL^{-1}.

Cultures were incubated at 37 °C for 48 h. Three hours prior to harvesting, 0.2 μg mL^{-1} of colchicin (Sigma, St. Louis, MO, USA) was added. As specified by the International Atomic Energy Agency (IAEA 2001), the cultivation was followed by hypotonic treatment with 0.075 M KCl (Sigma, St. Louis, USA), fixation with 3 : 1 methanol–glacial acetic acid, air-drying and staining with 5% Giemsa (Sigma, St. Louis, MO, USA). For each blood sample, tested material and duration of elution, 500 metaphases were analysed scoring the number of chromosome and chromatid breaks, as well as acentric fragments.

Comet assay

Prior to the ending of elution periods 0.8 mL of whole blood was introduced into 8.2 mL of F-10 HAM's medium (Sigma, St. Louis, MO, USA) without serum or mitotic activator. Cultures were treated with 8 μg, 4 μg or 2 μg of each root filling material for 48 h at 37 °C. Simultaneously, negative control cultures were treated with the same volume of saline solution (NaCl 0.9%–Sigma, St. Louis, MO, USA) whereas the positive controls were treated with methyl methanosulphonate (Sigma, St. Louis, MO, USA) for the last 3 h at a final concentration of 3 μg mL^{-1}.

The comet assay was performed according to a standard protocol (Singh *et al.* 1988). All the chemicals needed to perform the comet assay were obtained from Sigma (St. Louis, MO, USA). The culture medium was carefully removed, and 5 μL of the sedimented lymphocytes was suspended in 100 μL of 0.5% low melting agarose to obtain 10 000 of lymphocytes per slide. This agarose layer was sandwiched between a layer of 0.6% normal melting agarose and a top layer of 0.5% low melting agarose on fully frosted slides. The slides were coded and kept on ice during the polymerisation of each gel-layer. After the solidification of the 0.5% agarose layer, the slides were immersed in a lysis solution (1% sodium sarcosinate, 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-HCl, 1% Triton X-100 and DMSO 10%) at 4 °C. After 1 h, the slides were placed in an electrophoresis buffer (0.3 M NaOH, 1 mM Na_2EDTA, pH 13) at 0 °C for 20 min to allow the DNA to unwind. The electrophoresis was performed at 300 mA and 1.0 V cm^{-1} in a horizontal electrophoresis platform for 20 min. The slides were neutralised with a Tris-HCl buffer (pH 7.5) and stained with 10% ethidium-bromide for 10 min. Each slide was analysed using a Leitz Orthoplan epifluorescence microscope. A hundred comets per slide were analysed by the Comet assay II automatic digital analysis system (Perceptive Instruments Ltd, Halstead, UK) measuring tail length and tail intensity (% DNA). During the analysis, the edges and eventually damaged parts of the gel as well as debris, superimposed comets, comets of uniform intensity and comets without a distinct head ('clouds', 'hedgehogs' or 'ghost cells') were avoided. For each root filling material, amount tested and elution period 100 comets were scored.

Statistical analysis

Differences in the number of chromosomal aberrations between treated and control lymphocytes were evaluated using chi-square and Fisher's PLSD test. To test the differences in comet assay endpoints the Kruskall–Wallis non parametric test was used. The level of significance was set at 0.05.

Results

The trypan blue exclusion test revealed that 32 μg and 16 μg caused cytotoxicity higher than 30% (Fig. 1). Thus, they were excluded from further genotoxicity testing. Results on chromosomal aberration analysis are presented in Tables 2–5 and Fig. 2 according to the duration of the elution period. Results are presented as the median value of testings on three different blood samples. Fig. 3 shows the measured comet assay endpoints for all six tested root filling materials by the amount of material tested and elution duration. Of the zinc eugenol-based sealers Hermetic and SuperEBA, induced a significant increase in DNA migration ability (Fig. 3). The effect was observed only at the highest tested dose (0.8 μg mL^{-1}) and in a period up to 1 h after polymerization.

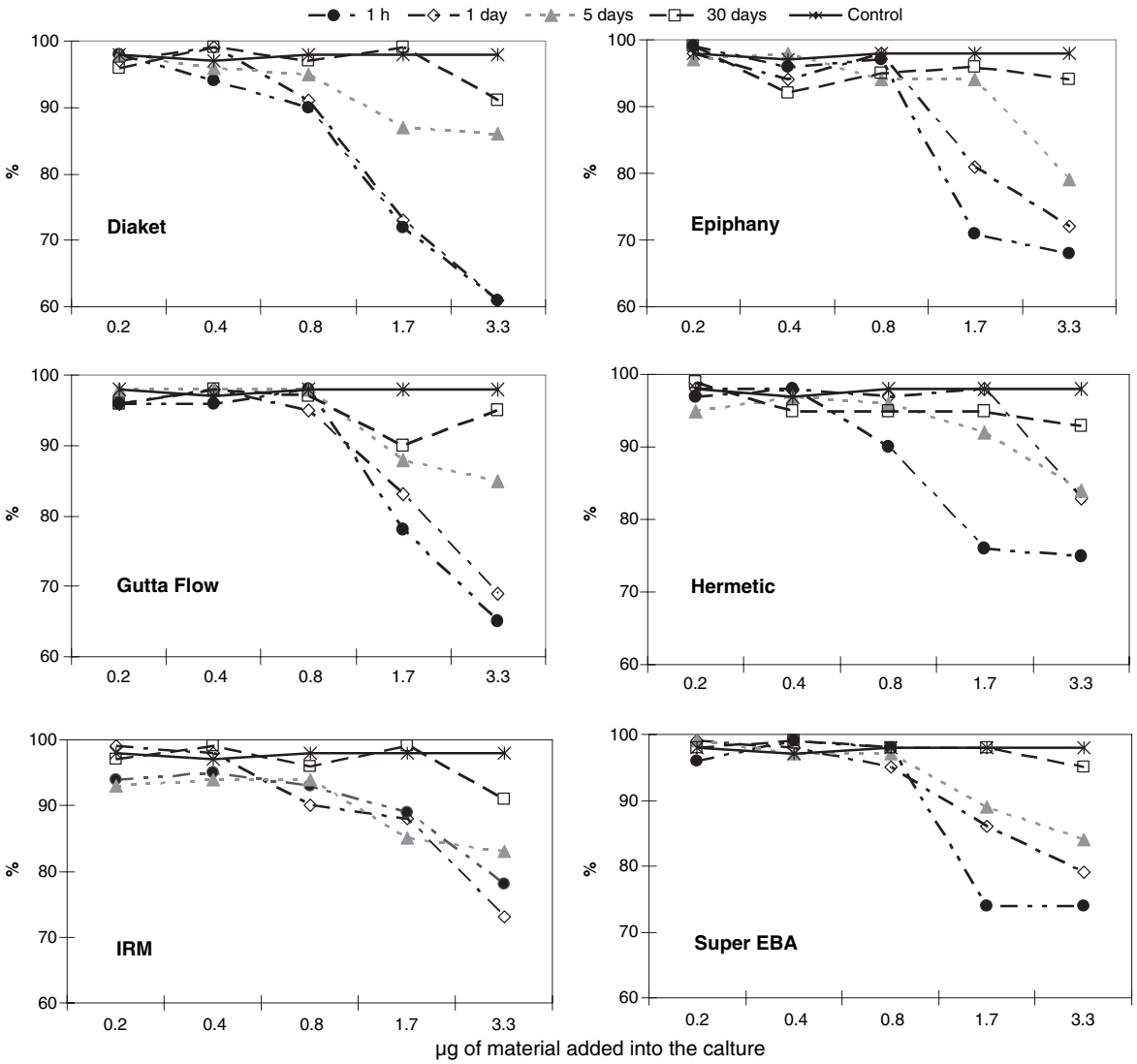

Figure 1 Viability of human lymphocytes using trypan blue exclusion test regarding to elution period of root filling materials (1 h, 1 day, 5 days and 30 days). A thousand lymphocytes were scored per each material and concentration used.

Table 2 Number of chromosomal aberration analysis in human lymphocytes treated with six root canal sealers after 1 h of elution

Root canal sealers	Concentration mL^{-1}	Number of aberrations		
		Chromatid break	Chromosome break	Acentric fragment
Control[a]	22 μL of 0.9% NaCl	3	0	0
Diaket	0.8 μg	6	4	0
	0.4 μg	3	2	0
	0.2 μg	3	1	0
Epiphany	0.8 μg	4	1	1
	0.4 μg	1	0	0
	0.2 μg	3	1	0
GuttaFlow	0.8 μg	4	1	0
	0.4 μg	2	0	2
	0.2 μg	2	0	0
Hermetic	0.8 μg	11	1	0
	0.4 μg	7	0	3
	0.2 μg	5	2	0
IRM	0.8 μg	5	1	0
	0.4 μg	4	1	0
	0.2 μg	3	0	0
SuperEBA	0.8 μg	7	2	0
	0.4 μg	3	2	0
	0.2 μg	4	0	0
Bleomycin[b]	30 μg	125**	47**	106**

A five hundred metaphases were scored per each material and concentration used.
[a]Negative control.
[b]Positive control, $^{**}P < 0.01$, $^{*}P < 0.05$ versus control.

Table 3 Number of chromosomal aberration analysis in human lymphocytes treated with six root canal sealers after 1 day of elution

Root canal sealers	Concentration mL^{-1}	Number of aberrations		
		Chromatid break	Chromosome break	Acentric fragment
Control[a]	22 μL of 0.9% NaCl	3	0	0
Diaket	0.8 μg	8	0	0
	0.4 μg	4	0	0
	0.2 μg	3	1	0
Epiphany	0.8 μg	4	1	0
	0.4 μg	1	0	0
	0.2 μg	0	1	1
GuttaFlow	0.8 μg	3	1	1
	0.4 μg	1	2	0
	0.2 μg	2	0	0
Hermetic	0.8 μg	5	0	0
	0.4 μg	5	1	0
	0.2 μg	3	0	0
IRM	0.8 μg	8	0	0
	0.4 μg	4	0	1
	0.2 μg	3	0	0
SuperEBA	0.8 μg	7	1	0
	0.4 μg	3	0	1
	0.2 μg	2	1	0
Bleomycin[b]	30 μg	108**	39**	84**

A five hundred metaphases were scored per each material and concentration used.
[a]Negative control.
[b]Positive control, $^{**}P < 0.01$versus control.

For the IRM only a slight increase of tail length was observed. In the first hour after polymerization only Hermetic at 0.8 μg mL^{-1} significantly elevated the number of aberrant cells (Fig. 2). The number of chromatid breaks was mostly affected. At the same period after polymerization SuperEBA at 0.8 μg mL^{-1}

Table 4 Number of chromosomal aberration analysis in human lymphocytes treated with six root canal sealers after 5 days of elution

Root canal csealers	Concentration mL^{-1}	Number of aberrations		
		Chromatid break	Chromosome break	Acentric fragment
Control[a]	22 μL of 0.9% NaCl	3	0	0
Diaket	0.8 μg	4	1	0
	0.4 μg	1	1	1
	0.2 μg	2	0	0
Epiphany	0.8 μg	0	1	1
	0.4 μg	3	0	0
	0.2 μg	2	0	0
GuttaFlow	0.8 μg	2	0	1
	0.4 μg	1	1	0
	0.2 μg	2	0	1
Hermetic	0.8 μg	5	0	0
	0.4 μg	3	0	1
	0.2 μg	2	0	0
IRM	0.8 μg	3	1	0
	0.4 μg	2	0	0
	0.2 μg	2	1	0
SuperEBA	0.8 μg	4	1	0
	0.4 μg	3	0	0
	0.2 μg	2	0	0
Bleomycin[b]	30 μg	113**	51**	94**

A five hundred metaphases were scored per each material and concentration used.
[a]Negative control.
[b]Positive control, $^{**}P < 0.01$ versus control.

Table 5 Number of chromosomal aberration analysis in human lymphocytes treated with six root canal sealers after 30 days of elution

Root canal sealers	Concentration mL^{-1}	Number of aberrations		
		Chromatid break	Chromosome break	Acentric fragment
Control[a]	22 μL of 0.9% NaCl	3	0	0
Diaket	0.8 μg	3	0	0
	0.4 μg	3	0	0
	0.2 μg	2	1	0
Epiphany	0.8 μg	1	0	1
	0.4 μg	2	1	0
	0.2 μg	1	1	0
GuttaFlow	0.8 μg	2	0	0
	0.4 μg	2	0	0
	0.2 μg	2	1	0
Hermetic	0.8 μg	2	0	0
	0.4 μg	3	1	0
	0.2 μg	2	1	0
IRM	0.8 μg	3	0	0
	0.4 μg	2	0	0
	0.2 μg	3	0	0
SuperEBA	0.8 μg	4	0	1
	0.4 μg	2	1	0
	0.2 μg	3	0	0
Bleomycin[b]	30 μg	124**	42**	110**

A five hundred metaphases were scored per each material and concentration used.
[a]Negative control.
[b]Positive control, $^{**}P < 0.01$ versus control.

revealed only a slight and statistically insignificant increase in the number of chromatid breaks. One hour after polymerization at the concentration of 0.8 μg mL^{-1} Diaket significantly increased comet assay parameters (Fig. 3). For the same period and concentration only a slight increase in chromatid

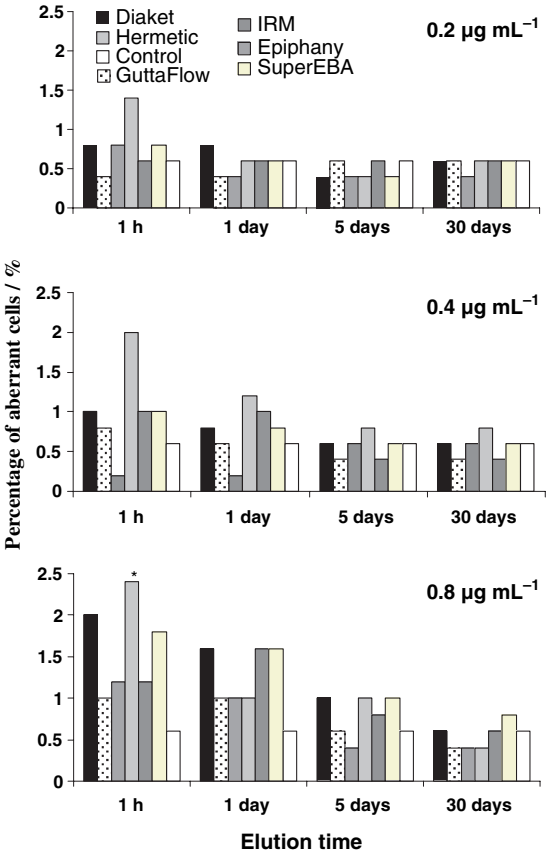

Figure 2 Percentage of aberrant cells in human lymphocyte cultures treated with six root canal sealers after different periods of elution. A five hundred metaphases were scored per each treatment procedure. Results for positive control: lymphocytes treated with 30 μg mL^{-1} of bleomycin; aberrant cells (AC) $_{1\ h}$ = 97; $AC_{1\ day}$ = 103; $AC_{5\ days}$ = 107; $AC_{30\ days}$ = 26.8; * $P < 0.05$ versus control.

breaks and number of aberrant cells was observed (Tables 2 and 3, Fig. 2).

After 24-h leakage only Diaket at the highest concentration tested (0.8 μg mL^{-1}) significantly elevated comet assay parameters (Fig. 3). The same concentration of Diaket exhibited only a slight effect on the increase of chromosomal aberrations.

After elutions longer than 1 day, no effect for any of the tested root canal on chromosomal aberration induction or DNA migration ability was observed.

Discussion

To avoid unwanted side effects following the use of canal sealers, which is important for the clinical outcome, only materials exerting minimum deleterious effects on living cells should be used. According to the literature, cyto/genotoxicity of some dental materials was dose dependent (Geurtsen & Leyhausen 1997, Briseno & Willershausen 1991, Eldeniz *et al.* 2007a, Bakopoulou *et al.* 2008). To implement the toxicity testing guidelines (Organisation for Economic Co-operation and Development (OECD 1997 and to test whether the dose dependent effect could be obtained with evaluated sealers, the cultures were treated with three different amounts of each material. Also, each material has to be examined by several *ex vivo* and *in vivo* tests (Heil *et al.* 1996, OECD 1997). One such test is chromosomal aberration analysis on mammalian cells, which was applied in this study. This technique is used for the evaluation of the genotoxic potential of chemical substances. Analysis of chromosomal aberrations (CA) revealed the frequency of chromatide breakages, chromosome breakages and accentric fragments in human peripheral blood lymphocytes. However, the technique does have limitations. It requires the cells to undergo cell division in order to visualize induced DNA damage. Furthermore, of all induced DNA strand breaks only those whose repair will result in the formation of aberrant chromosome morphology will be detected. The majority of DNA strand breaks that are successfully repaired will remain unnoticed (Obe *et al.* 2005). To overcome those limitations and to ensure that, if induced, a frequency of aberrations relevant to perform the evaluation will be recorded, 500–1000 metaphases have to be analysed. To test and demonstrate the ability of the technique to detect any genotoxic effect, each evaluation should include a treatment with a known clastogen as positive control. The use of chromosomal aberrations in evaluation of genotoxicity of dental materials was recommended by Geurtsen (2000) and Ribeiro (2008) in their review papers on biocompatibility testings. The method was successfully applied in evaluations of composites (Bakopoulou *et al.* 2008), adhesive systems (Prica *et al.* 2006), root filling materials (Geurtsen & Leyhausen 1997), and various agents used in dental practice (Hagiwara *et al.* 2006, Nishimura *et al.* 2008). The comet assay was also used as a more sensitive cytogenetic technique. The method detects the level of primary DNA damage in lymphocytes resulting from direct interaction of monomers leached from the material with DNA or due to oxidative stress induced in treated cells (Collins 2004).

In this study, using both CA analysis and comet assay, no genotoxic potential was noted for Epiphany

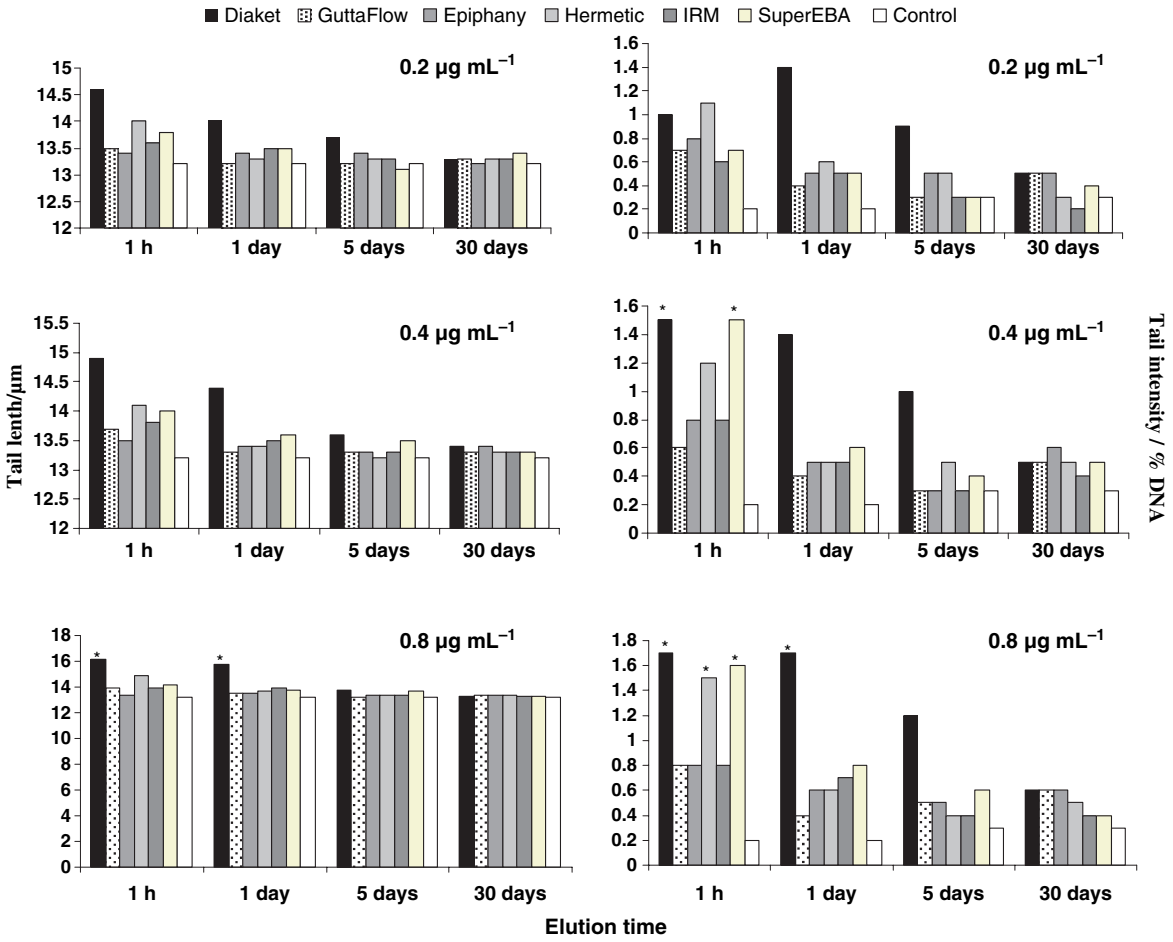

Figure 3 Comet assay end-points for human lymphocytes treated with six different root canal sealers after different periods of elution. A hundred micrographs were scored per each treatment procedure. Results for positive control: lymphocytes treated with 3 μg mL^{-1} of methylmethanosulphonate (MMS); $TL_{1\ h} = 32.37 \pm 1.26$, $TI_{1\ h} = 19.9 \pm 0.86$; $TL_{1\ day} = 31.9 \pm 1.21$, $TI_{1\text{-}day} = 18.7 \pm 0.79$; $TL_{5\ days} = 32.6 \pm 1.19$, $TI_{5\ days} = 19.2 \pm 0.81$; $TL_{30\ days} = 32.7 \pm 1.22$, $TI_{30\ days} = 19.7 \pm 0.86$; * $P < 0.05$ versus control.

or GuttaFlow. Eldeniz *et al.* (2007a,b) reported substantial toxicity for both materials evaluated on fibroblasts derived from human gingiva and L929 cell lines. However, the authors reported that GuttaFlow had the least toxic effect. In this study, doses below the materials' cytotoxicity were used to avoid misinterpretation of damage caused by toxicity as genotoxic lesions. Thus, at the level of exposure to Epiphany or GuttaFlow concentration of leached residual components are not capable of inducing DNA damage in the peripheral blood lymphocytes. According to the manufacturer's material safety data sheet, Epiphany contains UDMA, PEGDMA, EBPADMA and Bis-GMA have been shown to be genotoxic (Schweikl *et al.* 2001, Huang *et al.* 2003). According to the present results it could be suggested that the concentration of residual monomers that are leached from the polymerized material under the experimental conditions was too low to exhibit genotoxic activity.

Of the ZnOE-based sealers only Hermetic and SuperEBA at 0.8 μg mL^{-1} exhibited a limited genotoxic effect in the first hour after polymerization. Tai *et al.* (2001) reported that zinc-oxide eugenol-based root canal sealers exhibited significant genotoxic potential on cultured V79 (Chinese hamster lung fibroblasts) cells using DNA digestion and precipitation assays. Huang *et al.* (2001) detected an increased DNA migration ability in oral squamous cell carcinoma (OC2) cells

treated with several zinc oxide eugenol-based root canal sealers. However, it was not dose-dependent. Eugenol (4-allyl-2-methoxy phenol) is one of the components of those sealers that could be responsible for the observed genotoxic effects. The continuous leaching of eugenol from ZnOE sealers was observed by Fujisawa & Masuhara (1979). Hikiba *et al.* (2005) reported a significant increase in the chromosomal aberrations in Syrian Hamster Embryo Cells treated with eugenol. *In vivo* study on mice indicated that eugenol is capable of increasing micronucleus frequency in polychromatic erythrocytes (Ellahuene *et al.* 1994). By contrast, Chang *et al.* (2000) reported that on human fibroblasts eugenol exhibited cytotoxic rather than genotoxic activity, and Maura *et al.* (1989) failed to detect its effect at the chromosomal level using the bone marrow micronucleus test in rats. Zinc oxide itself was proved to be genotoxic in Syrian hamster embryo cells (Hikiba *et al.* 2005) and in Chinese hamster ovary cells (Dufour *et al.* 2006) significantly increasing the frequency of chromosomal aberrations. Differences in observed genotoxic effects between three tested ZnOE-based sealers may be due to their different polymerization efficiency, immobilization of Zn-containing compounds within the material after its polymerization, and differences in the content of additional compounds that may affect the level of cytotoxicity (Ørstavik 2005). Twenty-four hours after polymerization there were only slight differences in the genotoxic potential of ZnOE sealers. After 5-day elution no differences were observed between the tested materials. As reported, concentrations of zinc ions in the inorganic solvent due to its leaching from zinc-based materials varied from 0.26 μg mL^{-1} to 0.30 μg mL^{-1} (Czarnecka *et al.* 2003, Campus *et al.* 2007). Campus *et al.* (2007) reported that the concentration of eugenol released after 24 h of elution from ZnOE based sealers was 0.46×10^{-5} mol L^{-1}. As previously discussed, detected concentrations of $Zn^{2+}$and eugenol are able to exhibit cyto/genotoxic effects. Imazato *et al.* (2009) detected 400-50 μg mL^{-1} of HEMA, 100-10 μg mL^{-1} of TEGDMA in eluates of restoration materials. The authors also reported that the measured concentrations of monomers induced adverse effects in osteoblast-like cells. TEGDMA was proved to be cytotoxic already at the concentration of 500 μg mL^{-1} (Demirci *et al.* 2008). Thus, due to the process of leaching, a geno/cytotoxic concentration of a specific component in the treated cell culture may be reached. There were no significant differences in genotoxicity between the tested root sealers 1 h or 1 day after polymerization. In this study significant levels of DNA damage were observed only using the comet assay. As root sealers have been tested at concentrations that showed toxicity lower than 15%, the possible contribution of the cytotoxic effect to the increase in DNA migration ability was negligible. Although insignificant, the increase in the number of aberrant cells observed 1 h after polymerization point out its limited clastogenic effect. Such results suggest that induced DNA damage could be efficiently repaired and has no significant effect on genomic stability at the level of the chromosome morphology. Some previous studies have reported significant toxic activity of polyvinyl resin-based sealers in different cell types (Meryon & Brook 1990, Briseno & Willershausen 1991, Miletic *et al.* 2000, Asrari & Lobner 2003). Vinyl acetate used in root sealers as one of the copolymers is a proved mutagen and carcinogen (Bogdanffy & Valentine 2003). However, there is no published data on the leakage of that component from polymerized sealer or the release of residual copolymers after the placement.

Conclusion

Diaket, Hermetic, IRM and SuperEBA exhibited limited genotoxic activity on peripheral blood lymphocytes *ex vivo*. Since the effect was observed only for a period shorter than 5 days after polymerization and at the highest concentration tested (0.8 μg mL^{-1}) it should not pose a significant risk to the human genome. No effect of GuttaFlow or Epiphany on the frequency of chromosomal aberrations or comet assay endpoint was observed.

Acknowledgements

This investigation was supported by the Croatian Ministry of Science, Education and Sports as a part of the 'Themes No: 022-0222148-2137 and No: 065-0650444-0418).

References

Asrari M, Lobner D (2003) In vitro neurotoxic evaluation of root-end–filling materials. *Journal of Endodontics* **29**, 743–6.

Bakopoulou A, Mourelatos D, Tsiftsoglou AS, Mioglou E, Garefis P (2008) Sister-chromatid exchange, chromosomal aberrations and delays in cell-cycle kinetics in human lymphocytes induced by dental composite resin eluates. *Mutation Research* **649**, 79–90.

Bogdanffy MS, Valentine R (2003) Differentiating between local cytotoxicity, mitogenesis, and genotoxicity in carcinogen risk assessments: the case of vinyl acetate. *Toxicology Letters* **11**, 83–98.

Briseno BM, Willershausen B (1991) Root canal sealer cytotoxicity on human gingival fibroblasts: 2. Silicone- and resin-based sealers. *Journal of Endodontics* **17**, 537–40.

Campus G, Garcia-Godoy F, Gaspa L *et al.* (2007) Dependence of kinetic variables in the short-term release of Hg^{2+}, $Cu^{2+}$and Zn^{2+}ions into synthetic saliva from an high-copper dental amalgam. *Journal of Materials Science: Materials in Medicine* **18**, 1521–7.

Chang YC, Tai KW, Huang FM, Huang MF (2000) Cytotoxic and nongenotoxic effects of phenolic compounds in human pulp cell cultures. *Journal of Endodontics* **26**, 440–3.

Collins AR (2004) The Comet Assay for DNA damage and repair: principles, applications, and limitations. *Molecular Biotechnology* **26**, 249–61.

Czarnecka B, Limanowska-Shaw H, Nicholson JW (2003) Ion-release, dissolution and buffering by zinc phosphate dental cements. *Journal of Materials Science: Materials in Medicine* **14**, 601–4.

Demirci M, Hiller K-A, Bosl C, Galler K, Schmalz G, Schweikl H (2008) The induction of oxidative stress, cytotoxicity, and genotoxicity by dental adhesives. *Dental Materials* **24**, 362–71.

Dufour EK, Kumaravel T, Nohynek GJ, Kirkland D, Toutain H (2006) Clastogenicity, photo-clastogenicity or pseudo-photo-clastogenicity: Genotoxic effects of zinc oxide in the dark, in pre-irradiated or simultaneously irradiated chinese hamster ovary cells. *Mutation Research* **607**, 215–24.

Eldeniz AU, Erdemir A, Kurtoglu F, Esener T (2007a) Evaluation of pH and calcium ion release of Acroseal sealer in comparison with Apexit and Sealapex sealers. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics* **103**, e86–91.

Eldeniz AU, Mustafa K, Ørstavik D, Dahl JE (2007b) Cytotoxicity of new resin-, calcium hydroxide- and silicone-based root canal sealers on fibroblasts derived from human gingiva and L929 cell lines. *International Endodontic Journal* **40**, 329–37.

Ellahuene MF, Perez-Alzola LP, Orellana-Valdebenito M, Munoz C, Lafuente-Indo N (1994) Genotoxic evaluation of eugenol using the bone marrow micronucleus assay. *Mutation Research* **320**, 175–80.

Fujisawa S, Masuhara E (1979) The in vitro biological evaluation of dental cements using elution and binding by bovine serum albumin. *Shika Rikogaku Zasshi (Journal of the Japan Society for Dental Apparatus and Materials)* **20**, 142–50.

Geurtsen W (1998) Substances releases from non-amalgam dental filling materials. *European Journal of Oral Sciences* **106**, 687–95.

Geurtsen W (2000) Biocompatibility of resin-modified filling materials. *Critical Reviews in Oral Biology and Medicine* **11**, 333–55.

Geurtsen W, Leyhausen G (1997) Biological aspects of root canal filling materials-histocompatibility, cytotoxicity, and mutagenicity. *Clinical Oral Investigations* **1**, 5–11.

Gulati N, Chandra S, Aggarwal PK, Jaiswal JN, Singh M (1991) Cytotoxicity of eugenol in sealer containing zinc oxide. *Endodontics and Dental Traumatology* **7**, 181–5.

Hagiwara M, Watanabe E, Barrett JC, Tsutsui T (2006) Assessment of genotoxicity of 14 chemical agents used in dental practice: ability to induce chromosome aberrations in Syrian hamster embryo cells. *Mutation Research* **603**, 111–20.

Heil J, Reifferscheid G, Waldmann P, Leyhausen G, Geurtsen W (1996) Genotoxicity of dental materials. *Mutation Research* **28**, 181–94.

Hikiba H, Watanabe E, Carl Barrett J, Tsutsui T (2005) Ability of fourteen chemical agents used in dental practice to induce chromosome aberrations in syrian hamster embryo cells. *Journal of Pharmaceutical Sciences* **97**, 146–52.

Huang TS, Lee H, Kao CT (2001) Evaluation of the genotoxicity of zinc oxide eugenol-based, calcium hydroxide-based, and epoxy resin-based root canal sealers by comet assay. *Journal of Endodontics* **27**, 744–8.

Huang FM, Chou MY, Chang YC (2003) Dentin bonding agents induce c-fos and c-jun protooncogenes expression in human gingival fibroblasts. *Biomaterials* **24**, 157–63.

Imazato S, Horikawa D, Nishida M, Ebisu S (2009) Effects of monomers eluted from dental resin restoratives on osteoblast-like cells. *Journal of Biomedical Materials Research. Part B, Applied Biomaterials*, In Press.

International Atomic Energy Agency IAEA (2001) *Cytogenetic Analysis for Radiation Dose Assessment. A Manual* TechRepSer No 405. Vienna, Austria: International Atomic Energy Agency.

Kolokuris I, Katsaki-Kovatsi VP, Economides N, Poulololoulos A, Razos G, Vlemmas I (1998) Influence of zinc oxide and eugenol sealer on concentration of zinc, calcium and copper in rat tissues. *Endodontics and Dental Traumatology* **14**, 210–3.

Maura A, Pino A, Ricci R (1989) Negative evidence in vivo of DNA-damaging, mutagenic and chromosomal effects of eugenol. *Mutation Research* **227**, 125–9.

Meryon SD, Brook AM (1990) In vitro comparison of the cytotoxicity of twelve endodontic materials using a new technique. *International Endodontic Journal* **23**, 203–10.

Miletic I, Anic I, Karlovic Z, Marsan T, Pezelj-Ribaric S, Osmak M (2000) Cytotoxic effect of four root filling materials. *Endodontics and Dental Traumatology* **16**, 287–90.

Nencka D, Walia HD, Austin BP (1995) Histologic evaluation of the biocompatibilty of Diaket. *Journal of Dental Research* **74**, 101.

Newell DG (1998) Trypan blue exclusion method for cell viability estimation. In: Doyle A, Griffiths JB, eds. *Cell and Tissue Culture: Laboratory Procedures*. London, UK: John Wiley and Sons Ltd, pp. 77–82.

Nishimura H, Higo Y, Ohno M, Tsutsui TW, Tsutsui T (2008) Ability of root canal antiseptics used in dental practice to

induce chromosome aberrations in human dental pulp cells. *Mutation Research* **649**, 45–53.

Obe G, Pfeiffer P, Savage JRK *et al.* (2005) Chromosomal aberrations: formation, identification and distribution. *Mutation Research* **504**, 17–36.

Olsson B, Siwkowski A, Langeland K (1981) Intraosseus implantation for biological evaluation of endodontic materials. *Journal of Endodontics* **7**, 253–65.

Organisation for Economic Cooperation and Development. OECD (1997) *Genetic Toxicology: In Vitro Mammalian Cytogenetic Test* Guidelines for the Testing of Chemicals No. 473 Paris, France: OECD Publishing.

Ørstavik D (2005) Materials used for root canal obturation: technical, biological and clinical testing. *Endodontic Topics* **12**, 25–38.

Prica D, Galic N, Zeljezic D, Prica A (2006) Genotoxicity evaluation of five different dentin bonding agents by chromosomal aberration analysis. *Journal of Oral Rehabilitation* **33**, 462–71.

Ribeiro DA (2008) Do endodontic compounds induce genetic damage? A comprehensive review. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics* **105**, 251–6.

Schweikl H, Schmalz G, Spruss T (2001) The induction of micronuclei in vitro by unpolymerized resin monomers. *Journal of Dental Research* **80**, 1615–20.

Serene TP, Vesely J, Boackle RJ (1988) Complement activation as a possible *in vitro* indication of the inflammatory potential of endodontic materials. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics* **65**, 354–7.

Singh NP, McCoy MT, Tice RR, Schneider EL (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. *Experimental Cell Research* **175**, 184–91.

Spahl W, Budzikiewicz H (1994) Qualitative analysis of dental resin composites by gas and liquid chromatographyhass spectrometry. *Fresenius Journal of Analytical Chemistry* **350**, 684–91.

Spangberg L (1969) Biological effects of root-canal-filling materials. Part 4. Effect in vitro of water-soluble components of root-canal-filling materials on HeLa cells. *Odontologisk Revy* **20**, 289–99.

Stanley HR (1992) Local and systemic responses to dental composites and glass ionomers. *Advances in Dental Research* **6**, 55–64.

Tai KW, Huang FM, Huang MS, Chang YC (2001) Assessment of the genotoxicity of resin and zinc-oxide eugenol-based root canal sealers using an in vitro mammalian test system. *Journal of Biomedical Materials Research* **59**, 73–7.

Takahara K, Onodera A, Matsumoto K (1990) Toxicity of root canal sealers on rat bone cells in primary culture. *Endodontics and Dental Traumatology* **6**, 200–7.

Torabinejad M, Hong CU, Pitt Ford TR, Kettering JD (1995) Cytotoxicity of four root end filling materials. *Journal of Endodontics* **21**, 489–92.